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A Baculovirus Mutant Defective in PKIP, a Protein Which Interacts with a Virus-Encoded Protein Kinase

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We have found that a temperature-sensitive mutant of the baculovirus Ac*M*NPV, tsB97, is defective in PKIP, the product of ORF24 which was previously found to interact with and stimulate the activity of a virus-encoded protein kinase, PK-1 The mutant lacks the ability to form plaques and occlusion bodies at the nonpermissive temperature. The mutant displays several properties which suggest a defect in the latter half of the late phase of infection; these properties include a delay in the shutoff of host protein synthesis, the presence of aberrant electron-dense bodies associated with the virogenic stroma, and the production of few, if any, progeny budded virus. A study of the expression of selected late genes showed no difference in the timing or level of transcription or translation of most late genes. However, elevated levels of the late 6.9K protein, a protamine-like protein, were observed in mutant-infected cells at 24 h postinfection, suggesting a defect in the regulation of this protein. Two polypeptides, 40 and 6 kDa, exhibited considerably higher levels of steady-state phosphorylation in wt-infected cells versus tsB97-infected cells at 24 h p.i. and could be candidates for PK-1/PKIP-mediated phosphorylation. The tsB97 mutant also displayed a severe defect in very late gene transcription which accounts for its inability to form occlusion bodies. The effect of PKIP on very late gene transcription may be a secondary effect of the block in the late phase of infection. PKIP showed no ability to transactivate expression from a very late promoter in transient expression assays.

Key Words: Autographa californica nuclear polyhedrosis virus; temperature-sensitive mutant; protein kinase-interacting protein; PK-1; very late gene expression.

INTRODUCTION

Replication of *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) in cell culture is marked by three major phases of transcription, early, late, and very late (as reviewed in O'Reilly *et al.,* 1992). Early gene transcription takes place from 0 to 6 h postinfection (p.i.), apparently mediated by the host RNA polymerase II (reviewed by Friesen, 1997). The onset of late gene transcription depends on induction of a novel, α-amanitin-resistant RNA polymerase (Grula *et al.,* 1981; Huh and Weaver, 1990; Glocker *et al.,* 1992; Xu *et al.,* 1995; Beniya *et al.,* 1996) and viral DNA replication (reviewed by Lu and Miller, 1997). In transient expression assays in *Spodoptera frugiperda* (SF-21) cells, optimal expression from a late gene promoter requires 18 late expression factors (LEFs) (Todd *et al.,* 1995, 1996). Nine of these genes comprise the minimum requirement for efficient reporter plasmid replication or stability, while the remaining 9 *lefs* are likely to be acting at the level of either transcription or mRNA stablility (Lu and Miller, 1995). Very late gene expression in these transient assays is stimulated by an additional gene, *vlf-1* (very late expression factor 1) (Todd *et al.,* 1996), originally identified by analysis of a temperature-sensitive mutant defective in very late gene transcription and occlusion body formation (McLachlin and Miller, 1994). Attempts have been made to identify other *vlfs* in the Ac*M*NPV genome using the same transient expression assay, but none have been found, suggesting that *vlf-1* may act alone to transactivate very late promoters or that the present transient assay system has constraints in this type of search (Todd *et al.,* 1996).

In this paper, we describe a temperature-sensitive mutant, tsB97, which was observed to be defective in the formation of plaques and occluded virus at 33°C (Lee and Miller, 1979). Our analysis of this mutant showed impaired production of infectious budded virus at the nonpermissive temperature, as well as a delay in expression and a reduction in the level of two very late proteins, polyhedrin and P10. The defect in very late gene expression was found at the level of transcription. We have mapped this mutation to ORF24 of Ac*M*NPV as defined by the sequence and analysis of Ayres *et al.* (1994). Recently Fan *et al.* (1998) have shown using a yeast two-hybrid system that ORF24 encodes a protein which interacts with an Ac*M*NPV-encoded protein kinase, PK-1, and have designated this protein PKIP, protein kinase-interacting protein. The effect of a temperature-sensitive

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mutation in the PKIP gene *pkip* on protein phosphorylation during the infection process is also presented.

RESULTS

Phenotype of tsB97 mutant virus

Previous characterization showed that tsB97 has normal plaque formation and occlusion body production at 23°C but does not form plaques or occluded virus at the nonpermissive temperature of 33°C in SF-21 cells (Lee and Miller, 1979). We confirmed the phenotype of tsB97 at both 23 and 33°C using a plaque-purified isolate.

Mapping of the tsB97 mutation

Marker rescue experiments were performed to map the location of the mutation in tsB97. Viral DNA was cotransfected with individual clones from an overlapping set of genomic clones of wt Ac*M*NPV which together span the entire genome (Passarelli and Miller, 1993) and smaller subclones of the *Pst*I-I region depicted in Fig. 1A. Cells that had been cotransfected with mutant viral DNA and subclones of wt DNA were examined for occlusion-positive plaques. Those subclones showing rescue are depicted as thicker bold lines in Fig. 1A. Two genomic clones, BC5 and HL8, were able to rescue tsB97 DNA, with the number of occlusion-positive plaques being too numerous to count. The HL5 clone was not able to rescue; the lefthand end of HL5 was sequenced and begins within ORF26. Smaller plasmid subclones of this region, pBamHI-C, pPstI-I, and pHindIII-M, were tested and only pPstI-I was positive. A series of subclones of the *Pst*I-I region of Ac*M*NPV were tested for marker rescue, and clones which rescued are indictated by thick black lines (Fig. 1A). The smallest fragment tested to rescue tsB97 was a 1.7-kb *Hin*cII fragment (pPst-Hc 1.7) which provided on average 16 plaques per dish compared to less than 1 plaque per dish for tsB97 DNA alone.

Sequencing of Ac*M*NPV ORF24 and flanking regions

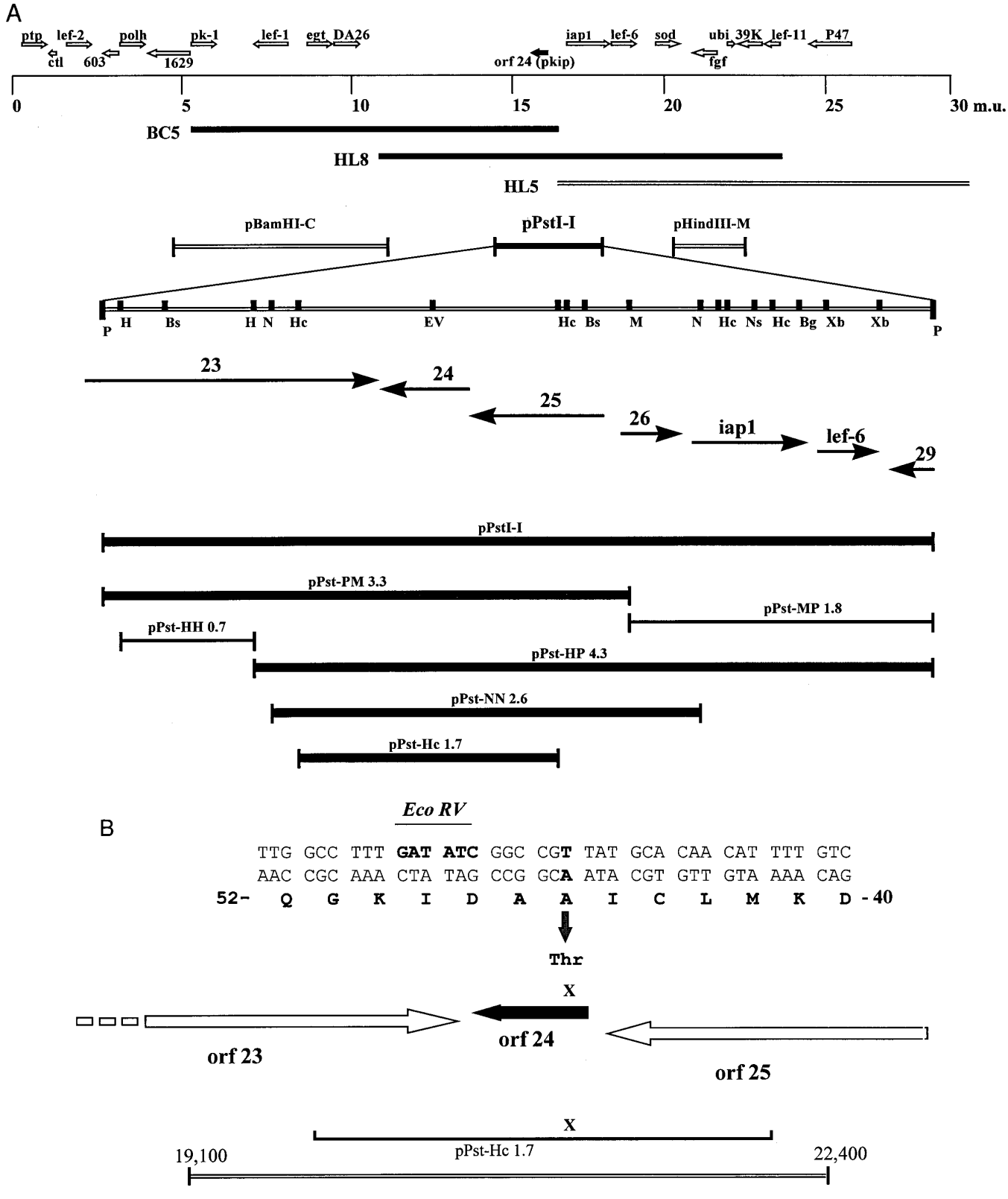
To sequence the mutant DNA in the region able to marker rescue tsB97, we subcloned the PstI-I fragment from tsB97. The region of the 1.7 kb *Hin*cII fragment of *Pst*I-I and additional flanking regions were sequenced on both strands for wt Ac*M*NPV and tsB97 viral DNA. The entire region sequenced is indicated by the double line at the bottom of Fig. 1B and extends from nucleotides 19,100 to 22,400 (Ayres *et al.,* 1994). We identified only a single nucleotide change in tsB97 that differed from the L1 strain of Ac*M*NPV, the parental virus strain from which the mutant was originally derived. The change from a G to A on the coding strand at nucleotide position 21,008 would result in a predicted codon change from alanine to threonine for ORF24 at amino acid position 46. There was one nucleotide difference noted between the wild-type L1 and C6 strains of Ac*M*NPV. At nucleotide position 20,289, L1 and tsB97 have an A compared to a T for the C6 strain of Ac*M*NPV. This difference is found at the third position of the codon for a valine residue near the C-terminus of ORF23 and does not cause an amino acid change.

Protein synthesis patterns in wt Ac*M*NPV- and tsB97-infected cells

The kinetics of protein synthesis in SF-21 cells infected with either wt Ac*M*NPV or the mutant virus tsB97 was examined. At the permissive temperature of 23°C, the pattern of protein synthesis was identical for both viruses, with normal levels of polyhedrin by 36 h postinfection (Fig. 2A). Marked differences were observed between wt- and tsB97-infected cells at 33°C (Fig. 2B). We have shown previously that the time course of Ac*M*NPV infection typically proceeds more rapidly at 33°C than at 23°C (McLachlin and Miller, 1994). This is also reflected by the higher levels of polyhedrin production by 24 h p.i. and the more rapid cessation of host protein synthesis in wt-infected cells at 33°C. Compared to wt-infected cells, tsB97-infected cells showed a slower progression of infection at 33°C, with both a delay in the shutoff of host protein synthesis and reduced levels of polyhedrin synthesis.

The synthesis of P10 was also affected by the tsB97 mutation, as shown by Coomassie blue staining. Expression of P10 at 23°C was similar for both wt- and tsB97-

FIG. 1. (A) Location of Ac*M*NPV genomic clones used in marker rescue of tsB97. Open reading frames (ORFs) found in this region of the genome which have a defined function are identified along the top by their common name. The large genomic fragments cloned into Lambda GEM-11 (Promega , Madison, WI) were sequenced to determine their end points. The BC5 clone extends from nucleotides 6627 to 21,442; HL8 extends from nucleotides 15,393 to 30,525; HL5 extends from nucleotides 22,060 to 38,451 (the numbers correspond to the published Ac*M*NPV sequence of Ayres *et al.,* 1994). The plasmid clone of PstI-I, which extends from nucleotides 18,880 to 24,116 (Ayres *et al.,* 1994) is expanded to indicate some of the restriction enzyme sites. Solid arrows show the position of ORFs within this region and the direction of the arrow indicates on which DNA strand the ORF is encoded. The names and relative positions of the plasmid subclones of PstI-I are shown by the solid black lines. Thick lines indicate clones which were positive for marker rescue of tsB97, and thin lines indicate plasmid clones which were negative for marker rescue of the mutant phenotype. Restriction endonuclease sites: Bg, *Bgl*I; Bs, *Bst*XI; EV, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; M, *Mlu*I; N, *Nru*I; Ns, *Nsi*I; P, *Pst*I; Xb, *Xba*I. (B) Schematic diagram to indicate the position of the pPstHc1.7 clone, which was positive for rescue of tsB97, relative to the predicted open reading frames. The region sequenced in both wt Ac*M*NPV (L1 strain) and tsB97 is indicated by the double black line. The site of the single nucleotide difference between wt and tsB97 is indicated by an X. Amino acids 40 to 52 of ORF24 are indicated in bold type below the sequence. The G to A transition on the coding strand, indicated by a C to T transition on the noncoding strand, alters the codon of amino acid 46 from an alanine (A) to a threonine (Thr). The position of the mutation in ORF24 is shown and marked by an X.


A
ptp
lef-2
ctl
603
polh
1629
pk-1
lef-1
egt
DA26
orf 24 (pkip)
iap1
lef-6
sod
fgf
ubi
39K
lef-11
P47
0
5
10
15
20
25
30 m.u.
BC5
HL8
HL5
pBamHI-C
pPstI-I
pHindIII-M
P H Bs H N Hc EV Hc Bs M N Hc Ns Hc Bg Xb Xb P
23
24
25
26
iap1
lef-6
29
pPstI-I
pPst-PM 3.3
pPst-MP 1.8
pPst-HH 0.7
pPst-HP 4.3
pPst-NN 2.6
pPst-Hc 1.7
B
Eco RV
TTG GCC TTT GAT ATC GGC CGT TAT GCA CAA CAT TTT GTC
AAC CGC AAA CTA TAG CCG GCA ATA CGT GTT GTA AAA CAG
52- Q G K I D A A I C L M K D -40
Thr
X
orf 23
orf 24
orf 25
X
pPst-Hc 1.7
19,100
22,400

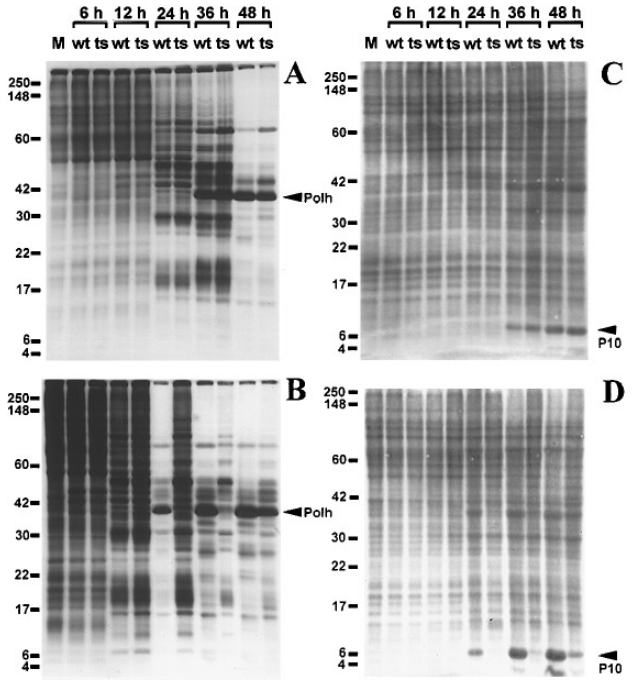


FIG. 2. Time course of protein expression in wt Ac*M*NPV- and tsB97-infected SF-21 cells. Infected cells were pulse-labeled for 1 h with a mixture of [^{35}S]methionine and cysteine at selected times postinfection. Total cellular protein was analyzed by SDS–PAGE on 12% gels, and labeled proteins were displayed by fluorography for cells infected at 23°C (A) or 33°C (B). The positions of MW markers are indicated on the left and the position of the polyhedrin protein (Polh) is shown by the arrowhead. Analysis of total protein in wt Ac*M*NPV- and tsB97-infected SF-21 cells was performed at 23°C (C) and 33°C (D). Total cellular proteins were analyzed by SDS–PAGE on 15% gels and stained with Coomassie blue. The position of P10 is indicated by an arrow. The time postinfection is indicated along the top of each gel. M, mock; wt, wild-type; ts, tsB97.

infected cells over the course of infection (Fig. 2C). In contrast, there were a dramatic delay in the onset of P10 accumulation and a reduction in the overall levels of P10 in cells infected by tsB97 at 33°C (Fig. 2D).

Northern blot analysis

We examined the effect of this mutation on the transcription of a selected group of genes normally expressed at late and/or very late times p.i. (Fig. 3). Total RNA was isolated from cells infected at 33°C with either wt or tsB97 virus at the indicated times and transcripts specific for *polh* were detected with a double-stranded probe which hybridizes to very late *polh* sense RNA as well as late antisense RNA. The levels of *polh* transcripts are dramatically reduced in the tsB97-infected cells and do not display the characteristic very late burst of transcription (Fig. 3A). The 3.8-kb transcript observed with this probe has components of both late antisense transcripts, which include ORF603 and ORF1629, and the very late sense transcript going through this region (Ooi and Miller, 1990). We used a another probe in an separate blot that was specific for ORF603 and did not observe any differences between wt- and tsB97-infected cells at 33°C for this late transcript (Ooi and Miller, 1990). On the other hand, the very late 13-kb *polh* transcript is expressed at low levels even after 48 h p.i. in the mutant-infected cells compared to wt-infected cells, which show abundant levels by 24 h p.i. and the typical burst of *polh* transcription between 24 and 48 h p.i.. This blot was stripped and reprobed with a probe specific for *vp39* (Fig. 3B). The VP39 capsid protein is encoded by a 2.2-kb transcript (Thiem and Miller, 1989) which is not decreased in the mutant-infected cells at 33°C. The levels of *vp39* RNA appear to be greater in the tsB97-infected cells, which may be a consequence of the mutation in this virus. It also indicates that the dramatic reduction in *polh*-specific transcripts cannot be due to loading lower amounts of RNA because Figs. 3A and 3B are the same Northern blot; a residual amount of *polh*-specific probe bound to the 1.3-kb transcript can be seen in the 48-h lane of wt-infected cells (Fig. 3B), which was due to incomplete stripping of the blot in Fig. 3A.

The levels of *p10* transcription were examined to further define the effect of this mutation on very late gene expression. At 33°C, we found that the *p10*-specific transcript of 0.72 kb, observed at 24 and 48 h p.i. in wt-infected cells, was absent in the tsB97-infected cells (Fig. 3C). The same RNA blot was stripped and used to study the effect of this mutation on transcription of *6.9K,* a gene transcribed in abundance during the late phase of infection (Wilson *et al.,* 1987). A 6.9K cDNA probe was used and there was no reduction seen in 6.9K transcripts

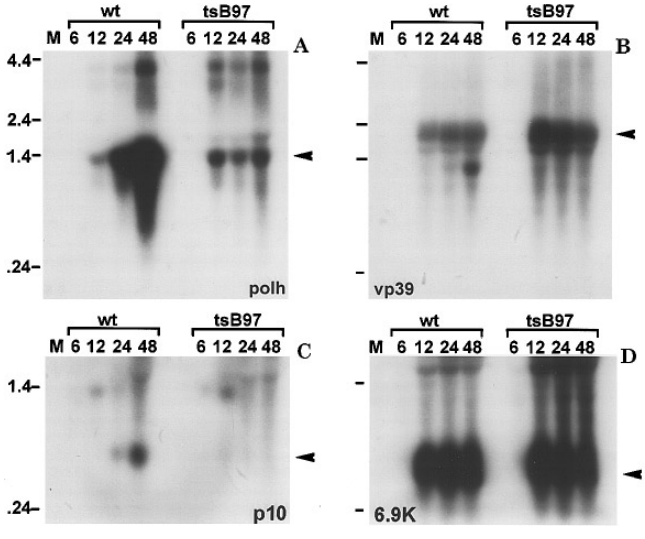


FIG. 3. Northern blot analysis of transcripts from wt Ac*M*NPV- or tsB97-infected SF-21 cells at 33°C. Total cellular RNA was collected from cells at various times p.i. as indicated at the top of each lane. The position of size standards for RNA is indicated on the left (0.24–9.5 kb RNA ladder, GIBCO/BRL). Probes specific for *polh* (A), *vp39* (B), *p10* (C), and *6.9K* (D) are described in detail under Materials and Methods. Arrowheads at the right of each panel point to the major transcripts for each gene analyzed.

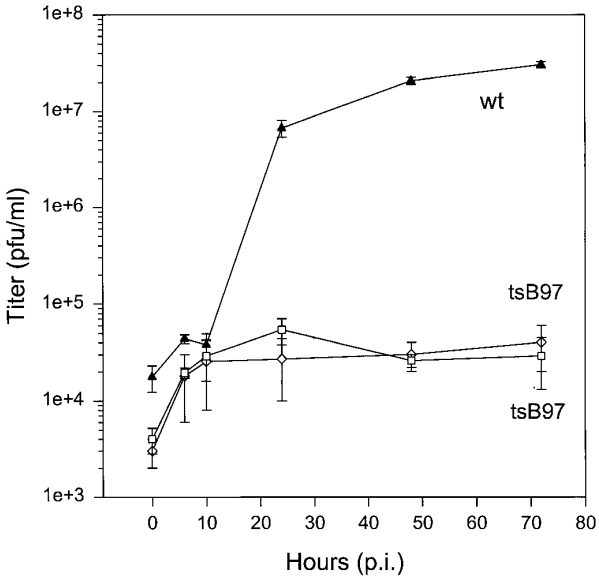


FIG. 4. Rate of release of infectious budded virus from SF-21 cells infected by wt Ac*M*NPV or mutant tsB97 at 33°C. Virus was collected from the cells at 0, 6, 10, 24, 48, and 72 h postinfection and then titered on SF-21 cells adapted for growth at 23°C. Two separate plates were infected with tsB97 and the titers for both wt AcMNPV- and tsB97-infected cells represent the mean ± SE for three determinations.

in tsB97-infected cells with respect to the timing and level of expression. Thus, the tsB97 mutation decreases transcription of the two major very late genes, *polh* and *p10,* but does not appear to decrease the transcription of at least three late genes, *603* ORF, *vp39,* and *6.9K.*

Release of infectious budded virus at 33°C

In contrast to a previous report (Lee and Miller, 1979), we found a dramatic decrease in the production of infectious budded virus from SF-21 cells infected with tsB97 at 33°C. The time course of infectious virus production at 33°C is illustrated for two plates of tsB97-infected cells and a plate of cells infected with wt Ac*M*-NPV. For cells infected with wild-type virus, both the rate of release and maximal levels of virus produced by the very late phase in infection were normal (Fig. 4). In contrast, release of infectious virus was severely restricted in the tsB97-infected cells, with the mean titer from samples taken during the very late phase not exceeding 2.0×10^4 PFU/ml. We do not attribute this simply to a delay in budded virus production because the levels of infectious virus in the medium were similar from 10 to 72 h p.i. compared to the earlier time points assayed. From this analysis, tsB97 can be reassigned to the class of ts mutant viruses that are defective in the production of budded virus at the nonpermissive temperature.

Electron microscopic analysis of tsB97-infected cells

To further address the effect of this mutation on virus production we examined mock-infected and wt- and tsB97-infected cells at 16 h p.i. by electron microscopy (Fig. 5). Mock-infected cells grown at 33°C displayed ultrastructural features typical of uninfected cells (data not shown). For cells infected with wt Ac*M*NPV, the nuclei contained a distinct virogenic stroma with multiple nucleocapsids throughout (Fig. 5A). With the exception of a rare cell, tsB97-infected cells also contained a distinct virogenic stroma with multiple nucleocapsids. However, a distinctive and universal feature of tsB97-infected cells was the additional presence of large, irregularly shaped, electron-dense bodies associated with the virogenic stroma (Figs. 5B and 5C). Figure 5C is a higher magnification of the same cell nucleus shown in Fig. 5B.

Budded virus production

To test the possibility that budded virus is produced by tsB97-infected cells at the nonpermissive temperature but is not infectious, medium was collected at 40 h p.i. from wt- and mutant-infected cells at 33°C and budded virus was purified through a sucrose cushion. Samples were analyzed by Western blot analysis using the polyclonal anti-VP39 antibody (Fig. 6). As negative controls, samples were prepared in the same fashion from mock-infected cells and cells that had been infected with either wt or tsB97 virus in the presence of aphidicolin to inhibit virus replication. Compared to the levels of budded virus detected from wt-infected cells (Fig. 6, lane 1) there was a significant reduction in the amount of virus in the medium from cells infected with tsB97. These data along with the observation of nucleocapsids within tsB97-infected cell nuclei suggest a defect in the movement of nucleocapsids out of the nucleus or a defect in budding from the plasma membrane.

Expression of VP39 and VLF-1 in tsB97-infected cells

We examined the levels of the major capsid protein produced in tsB97-infected cells at 33°C by Western blot analysis using an antibody to VP39 (Fig. 7A). By comparison to wt-infected cells, there was no difference observed in the levels or timing of capsid protein production. In both wt- and mutant-infected cells, VP39 was detected at similar levels beginning at 12 h p.i. through the very late phase of infection.

Because this mutant phenotype is occlusion-negative at the nonpermissive temperature and there is a reduction in transcription of two very late genes, *polh* and *p10,* we tested the possibility that the regulation of *vlf-1* expression might also be affected. Western blot analysis was done using a polyclonal antibody against VLF-1 to monitor the onset and temporal expression of *vlf-1* during infection at 33°C (Fig. 7B). A polypeptide (or closely spaced doublet) of approximately 52 kDa was detected initially at 12 h p.i. in both wt- and tsB97-infected cells and was expressed at similar levels for both groups of infected cells through the very late phase of infection.

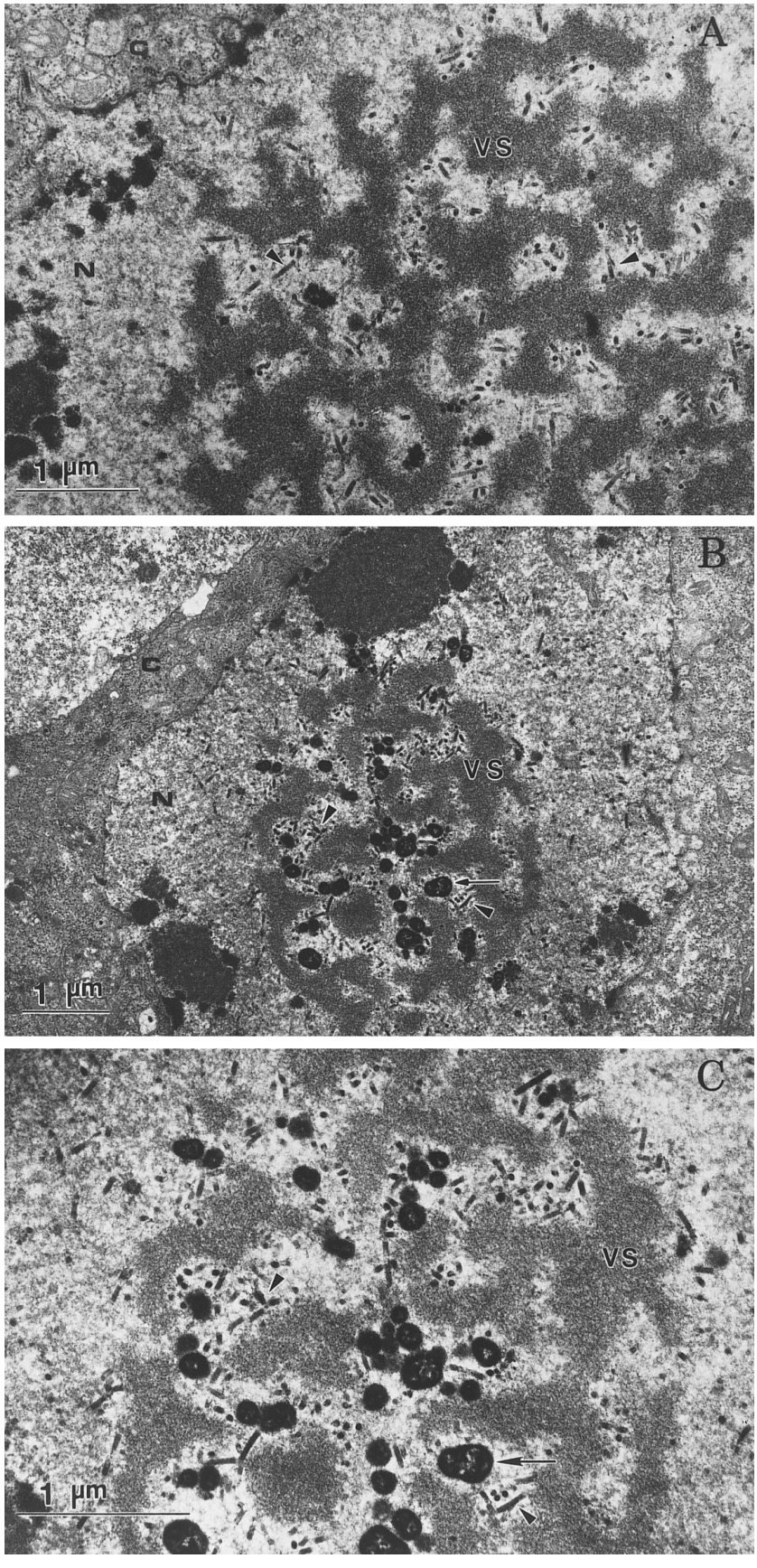


FIG. 5. Electron micrographs of (A) wt Ac*M*NPV- or (B and C) tsB97-infected SF-21 cells at 33°C. Cells were collected at 16 h p.i., fixed, sectioned, and stained for EM analysis. N, nucleus; C, cytoplasm; VS, virogenic stroma. Scale bars equal 1 μm.

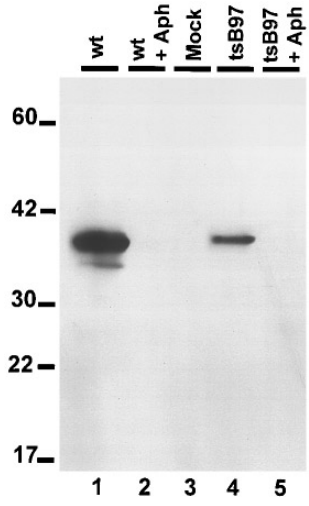


FIG. 6. Western blot analysis of budded virus production in wt Ac*M*NPV- and tsB97-infected SF-21 cells at 33°C. Medium was collected from wt-, tsB97-, or mock-infected cells at 40 h p.i., cells were pelleted, and the budded virus from the supernatant was collected by centrifugation through a 25% sucrose cushion. Virus was resuspended in 200 μL of PBS (pH 6.2) and 50 μL was mixed with an equal volume of 2× SDS loading buffer before Western analysis using an anti-VP39 polyclonal antibody. In some cases cells were infected in the presence of aphidicolin (Aph, 5 μg/ml) and medium was collected at 40 h p.i. as a negative control.

This protein was not detected in either mock-infected cells or infected cells at 6 h p.i. (Fig. 7B). There was a smaller polypeptide which was detected during the very late phase of infection: other data (not shown) indicate that this is a breakdown product of VLF-1 which occurs during sample preparation or storage.

Transient expression assay to test effect of ORF24 on very late gene expression

Since *vlf-1* expression does not appear to be affected by the tsB97 mutation, we tested the possible effect of ORF24 on very late gene expression using a transient assay system and a CAT reporter plasmid under the control of the *polh* promoter. The reporter plasmid phcwt contains sequences flanking the *polh* gene including the *pk-1* gene. This assay compared the levels of expression of the reporter gene in the presence of a clone library representing the entire Ac*M*NPV genome (Fig. 8, lane 2) to a library lacking lambda clones in the ORF24 region, BC5, HL8, and HL5 (see Fig. 1A) (Fig. 8, lane 3), and replacing those lambda clones with plasmids expressing LEFS within the region. Addition of *lef-1, lef-6, 39K, lef-11,* and *p47* (Fig. 8, lane 4) restored expression to a level similar to that observed with the Ac*M*NPV library (Fig. 8, compare lanes 2 and 4). No additional stimulation of expression was observed from the *polh* promoter when ORF24 was added; we tested plasmids in which ORF24 was expressed from its native promoter (ORF24) or from the late 6.9K gene promoter (6.9kORF24). This is in contrast to the stimulation observed with VLF-1. When the lambda clones expressing *vlf-1* were removed (PstH5 and HC9, lanes 7–9) and *vlf-1* was expressed in trans from either its native promoter (VLF-1) or from the 6.9K promoter (6.9kVLF-1) (Fig. 8, lanes 8 and 9), we observed a significant increase in CAT expression compared to a negative control in which *vlf-1* was supplied in an inactive form using a plasmid with a frame-shift mutation in *vlf-1* (VLF-1 fs, lane 7). *Lef-3* normally supplied by PstH5 and HC9 was included in all transfections for lanes 7–9. Thus, unlike VLF-1, ORF24 does not transactivate expression from the *polh* promoter in this transient assay system.

Protein phosphorylation

To examine the possible effect of this mutation in PKIP on protein phosphorylation, cells infected at 33°C with either wt or tsB97 virus were pulse-labeled in the presence of [^{33}P]orthophosphoric acid at selected times during infection. Coomassie blue staining of the protein profiles from a 1-h pulse-labeling experiment confirmed the temperature-sensitive phenotype, demonstrating a dramatic reduction in the steady-state levels of both polyhedrin (POLH) and P10 at 24 and 48 h p.i. in cells infected with the tsB97 virus (Fig. 9A). The same gel was subjected to fluorography to reveal the phosphoproteins which had been phosphorylated within the 1-h labeling period (Fig. 9B). The ts-mutant-infected cells contained

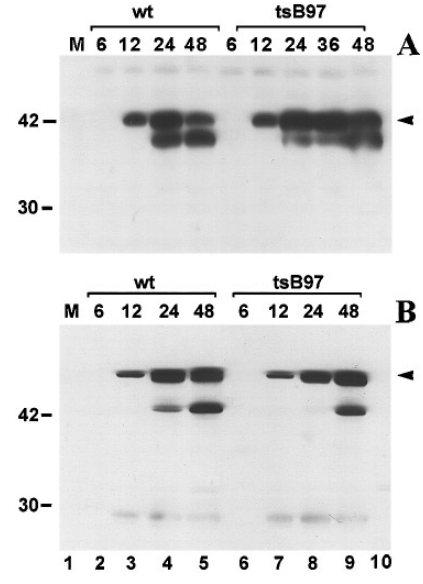


FIG. 7. Western blot analysis of VP39 and VLF-1 production in wt Ac*M*NPV- and tsB97-infected SF-21 cells at 33°C. Protein lysates made from either mock-infected (M) or virus-infected cells at the indicated time points (h p.i.) were separated using 12% SDS–PAGE, transferred to membrane (Immobilon-P) , and probed with either a polyclonal VP39 antibody (A) or a polyclonal VLF-1 antibody (B). Molecular weight markers are shown on the left side. The position of full-length VP39 and VLF-1 on each blot is indicated by an arrowhead at the right. M, mock-infected cells; wt, wild-type.

much higher levels of a phosphoprotein of approximately 13 kDa at 24 and 48 h p.i. than wt-infected cells (Fig. 9B). This protein is likely to be the phosphorylated form of 6.9K which has an apparent molecular size of 13 kDa when run on 12% SDS–polyacrylamide gels. For cells labeled for 1 h, there were no other apparent differences in the phosphoprotein profiles between wt- and tsB97-infected cells.

To determine whether the 13-kDa phosphoprotein was the 6.9K protein, proteins from wt- or mutant-infected cells were examined by Western blot analysis using an anti-6.9K antibody, raised to a synthetic peptide of 6.9K (Fig. 9C). Using the same infection conditions as those of Figs. 9A and 9B, we observed the same pattern of 6.9K expression by Western blotting. The steady-state levels of 6.9K protein detected at 24 and 48 h p.i. were much higher in tsB97-infected cells than in wt Ac*M*NPV-infected cells. The difference was most notable at 24 h p.i.. The level of the 6.9K protein declined between 24 and 48 h p.i. in both wt- and mutant-infected cells.

Profiles of phosphoproteins labeled for a 6-h time period did not show as dramatic a difference in phosphorylated 6.9K protein during the course of infection, although slightly higher levels of phosphorylated 6.9K were observed in mutant-infected cells from 12 to 52 h p.i.. Since the effect on 6.9K is most pronounced at 24 h in Figs. 9B and 9C, the 6-h labeling period used here may have failed to reveal the transient increase in phosphorylated 6.9K at 24 h p.i. (Fig. 9D). However, two phosphoproteins, 6 and 40 kDa, were observed in wt-infected cells but were reduced or absent in tsB97-infected cells. The identity of these proteins is not known.

Rescue of tsB97 mutant virus

To confirm that marker rescue of the tsB97 mutant virus was sufficient to restore a normal phenotype and that no other mutations in tsB97 were contributing to the observed phenotype, we isolated several occlusion-positive plaques from a direct transfection of tsB97 viral DNA and pPstHc17. We were able to amplify the rescued virus directly from plaques on cells propagated at 33°C which would require normal levels of infectious budded virus and these viral isolates were assayed again for evidence of the ts phenotype. At the nonpermissive temperature, all infected cells produced polyhedra indicating that the primary defect in occlusion body production was rescued by homologous recombination with ORF24 sequences.

DISCUSSION

We have found that a point mutation at the 46th codon of Ac*M*NPV ORF24 results in a temperature-sensitive phenotype characterized by defects in both budded virus production and very late gene transcription. The product of ORF24 was recently found to interact with and stimulate the *in vitro* activity of a virus-encoded protein kinase, PK-1, and the name PKIP, for protein kinase-interacting protein, was proposed for the ORF24 product (Fan *et al.,* 1998). We have examined the phenotype of the ts mutant in detail to determine where and how PKIP might function in the viral infection process.

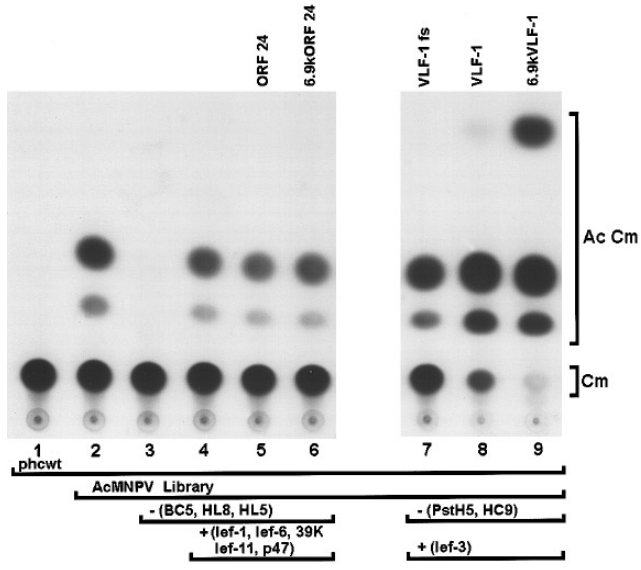


FIG. 8. Effect of ORF24 and VLF-1 on expression from the *polh* promoter. The reporter plasmid containing the CAT gene, phcwt, was transfected alone (lane 1) or cotransfected with the entire Ac*M*NPV clone library (lane 2) or the same library lacking clones BC5, HL8, and HL5 to remove ORF24 (lanes 3–6). Alternatively, the cells were transfected with phcwt and the same library of Ac*M*NPV clones lacking PstH5 and HC9 to remove VLF-1 (lanes 7–9). Essential lefs in each region were restored with the addition of *lef-1, lef-6, 39K, lef-11,* and *p47* (lanes 4–6) or the addition of *lef-3* (lanes 7–9). The effect of PKIP on expression of CAT was tested by cotransfecting ORF24 under control of its native promoter (pORF24, lane 5) or under the control of the 6.9K promoter (p6.9kORF24, lane 6) compared to *vlf-1* expressed from its native promoter (pVLF-1, lane 8) or *vlf-1* driven by the 6.9K promoter (p6.9kVLF-1, lane 9). As a negative control for *vlf-1* expression, a plasmid containing a frame shift in *vlf-1* (VLF-1 fs) was transfected (lane 7). CAT assays were performed on cell lysates harvested at 72 h posttransfection. The acetylated products (Ac Cm) and unacetylated substrate (Cm) are indicated to the right. Refer to Materials and Methods to cross-reference the plasmids used in this experiment.

The synthesis of most late proteins appear to be unaffected in tsB97-infected cells at 12 h p.i. as reflected by normal protein profiles at 12 h p.i. (Fig.2B). Furthermore, transcription of selected late genes is not decreased or temporally altered (Fig. 3), and normal timing and levels of selected late proteins, VP39 and VLF-1, are observed (Fig. 7). However, steady-state levels of the basic 6.9K protein were slightly elevated at 12 h p.i. in tsB97-infected cells and, by 24 h p.i., the levels of 6.9K were strikingly different in wt- and tsB97- infected cells (Fig. 9C).

The tsB97 defect became prominent during the period between 12 and 24 h p.i which is normally marked by a dramatic shutoff of host protein synthesis and the initia-

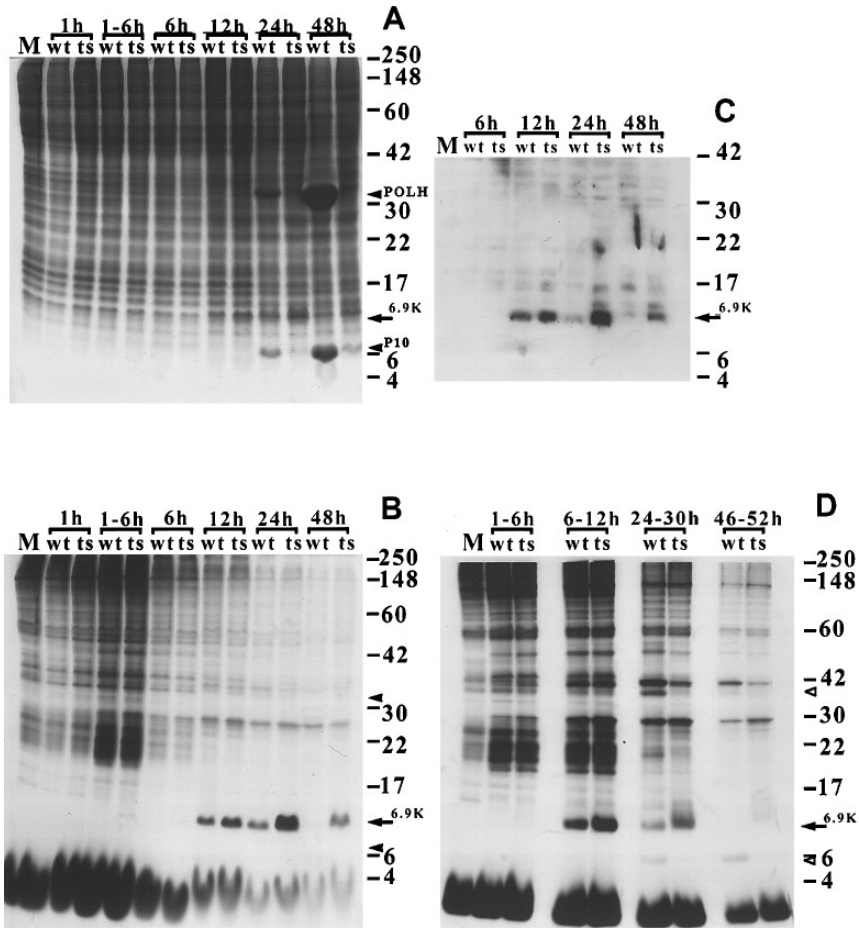


FIG. 9. Phosphoprotein profiles of wt- and tsB97-infected cells at 33°C. SF-21 cells were mock-infected (lane M) or infected with wt Ac*M*NPV (wt) or tsB97 (ts) at an m.o.i. of 10 and, at selected times p.i., cells were labeled with [^{33}P]orthophosphoric acid for 1 h (B) or 6 h (D). Both groups were starved for 1 h prior to the start of the labeling period. Total cell lysates from each time point were analyzed by SDS–PAGE (12% polyacrylamide). The same gel used for fluorography of the ^{33}P-labeled proteins shown in (B) was first stained with Coomassie blue to show the ts phenotype and the relative amounts of protein loaded in each lane (A). The positions of two very late proteins (POLH, P10) in wt-infected cells are indicated by solid arrowheads in A and B, while the position of the 6.9K protein is indicated by an arrow in all panels. The position of the two phosphoproteins that are either reduced or absent in tsB97-infected cells following a 6-h labeling period are indicated by open arrows (D). Western blot analysis of samples from a time course of infection at 33°C (C) using an anti-6.9K antibody. The positions and sizes (in kilodaltons) of protein markers are indicated at the right.

tion of very late gene expression (Fig. 2B). In the case of tsB97, the shutoff of host protein synthesis was delayed until 24 to 36 h p.i., while the synthesis of polyhedrin was delayed until 48 h p.i. Thus, there was a profound change in the timing of events during the latter half of the late phase which may, in turn, have delayed or disrupted the initiation of the very late phase of infection. The observation that tsB97-infected cells fail to exhibit the normal burst of very late gene transcription (Fig. 3) and fail to produce occlusion bodies could be due to the failure of the infection to progress beyond some step in the latter half of the late phase.

A defect during the latter half of the late stage of infection is also manifested in the failure of tsB97 to produce infectious progeny budded virus. Examination of tsB97-infected cells at 16 h p.i. revealed the presence of a virogenic stroma and nucleocapsids associated with the stroma. Whether these nucleocapsids were "normal" could not be fully assessed by microscopy, although major structural aberrations were not detected. A very noticeable feature of the virogenic stroma of tsB97-infected cells was the presence of aberrant electron-dense bodies which were not present, at least not in abundance, in the stroma of wt-infected cells. Similar bodies have been previously reported for a ts mutant of Ac*M*-NPV, tsN1054, which is also defective in budded virus production but maps to ORF54 and produces occlusion bodies (Olszewski and Miller, 1997). Thus, the observed aberrations in the tsB97 virogenic stroma and the defect in budded virus production do not necessarily account for the defect in very late gene expression observed in tsB97-infected cells. The aberrations in the virogenic stroma and the defect in budded virus formation, however, do implicate PKIP in a critical step during the latter

portion of the late phase. This defect could result in a "stalling" of the infection process in the late phase.

We have taken several steps to explore the possibility that PKIP has an additional, direct role in very late gene transcription but have obtained no evidence to support this view. We found that the timing and level of VLF-1 expression in tsB97-infected cells is normal. VLF-1 is a factor which is directly involved in very late gene transcription based on the phenotype of tsB837, a mutant defective only in very late gene expression (McLachlin and Miller, 1994), and the ability of VLF-1 to transactivate expression from very late promoters in transient expression assays (Todd *et al.,* 1996). We cannot formally exclude the possibility that VLF-1, although present, is not active in tsB97-infected cells. However, PKIP does not appear to be required for very late gene expression in transient expression assays even though PK-1 was supplied in the assay nor does PKIP appear to be required for the "activation" of VLF-1 in these assays (Fig. 8). Clearly these transient assays may not reflect all the parameters of very late gene expression during a normal infection process but the failure of PKIP to show any activity in these assays, coupled with the ability of very late gene transcription to occur in its absence, suggests an indirect rather than a direct effect of PKIP on very late gene transcription.

To examine the possibility that phosphorylation is affected in PKIP mutant-infected cells, we compared the phosphoproteins found in wt- and tsB97-infected cells. With a short (1-h) pulse of radiolabeled phosphate, we observed a difference in the level of only one phosphoprotein which we identified as the 6.9K protein. Surprisingly, we found that the difference in the levels of phosphorylation of this protein mirrored the difference in the steady-state levels of this protein. Thus, there is no evidence for a difference in the phosphorylation rate of this protein in tsB97-infected cells but rather there appears to be a difference in the rate of the accumulation of this protein. The steady-state levels of this protein normally decline from 12 to 24 h p.i but, in tsB97-infected cells, the 6.9K protein continues to accumulate during this period. By 24 h p.i., the difference in the level of the 6.9K protein in wt- versus tsB97-infected cells is substantial and this difference persists through 48 h p.i., although the levels decline in both cases during this period. The 6.9K protein, a protamine-like protein which is thought to associate with viral DNA in the core of the virion, is known to be phosphorylated in infected cells but becomes dephosphorylated during the assembly of nucleocapsids (Funk and Consigli, 1993). Because the 6.9K protein is a component of budded virions, it is possible that elevated steady-state levels of 6.9K in tsB97-infected cells is due to the failure of nucleocaspsids to bud from the cell and the accumulation of nucleocapsids within the cell. However, a similar difference in the level of the major capsid protein, VP39, was not observed. Furthermore, elevated levels of phosphorylated 6.9K protein were observed, a form which is apparently not found in mature virions. More work will be required to determine whether the effect of PKIP on the steady-state levels of 6.9K is related to the phosphorylation of 6.9K and whether differences in the levels of 6.9K are a primary or secondary consequence of the defect in PKIP. If a decline in 6.9K levels is associated with the initiation of the very late phase, the continued presence of 6.9K protein in tsB97-infected cells could explain the delay in initiating the very late phase.

A longer (6 h) phospholabeling period revealed the presence of two differentially phosphorylated polypeptides in wt-infected versus tsB97-infected cells. These phosphorylated polypeptides, 40 and 6 kDa, have not been further identified. They are preferentially found or preferentially phosphorylated in wt-infected cells; if these two polypeptides are substrates of PK-1, this result would be consistent with a temperature-sensitive defect in PKIP stimulation of PK-1 activity. These two polypeptides are observed to be phosphorylated at 24 h p.i. and differences in their phosphorylation could reflect the basis for the delay in the initiation of the very late phase of infection.

Temperature-sensitive mutants of PK-1 have also been isolated (Fan *et al.,* 1996) and, like tsB97, display a reduction in budded virus production and a block in very late gene expression. Examination of the nature of phosphoproteins in PK-1-mutant-infected cells might confirm the relationship between PKIP and PK-1 *in vivo* and lead to additional insights into the roles of PK-1 as well as the 40- and 6-kDa proteins during baculovirus infection.

MATERIALS AND METHODS

Virus and cells

A *S. frugiperda* (fall armyworm) cell line (Vaughn *et al.,* 1977), IPLB-SF-21 (SF-21), was adapted for growth at either 23 or 33°C in TC-100 medium (GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 0.26% tryptose broth (DIFCO), 100 U/ml penicillin G-Na salt, 0.2 mg/ml streptomycin sulfate, and 0.5 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO). The temperature-sensitive mutant tsB97 was originally isolated by treatment of wild-type (wt) Ac*M*NPV (L1 strain) with 5-bromodeoxyuridine and partially characterized by Lee and Miller (1979).

Plasmid construction

Plasmids pBamHI-C and pHindIII-M were constructed by cloning the *Bam*HI-C and *Hin*dIII-M fragments of Ac*M*NPV into the *Bam*HI or *Hin*dIII site, respectively, of pBR322. The plasmid pBSP-PstI was constructed by digesting wt Ac*M*NPV with *Pst*I and isolating the *Pst*I-I fragment (corresponds to nucleotides 18,880–24,116; Ay-

res *et al.,* 1994). This fragment was cloned into pBluescript(M13$^+$) (Stratagene, La Jolla, CA) at the *Pst*I site. Subclones of this plasmid were cloned for the purpose of marker rescue experiments and included pPst-PM3.3 (*Pst*I–*Mlu*I; nt 18,800–22,243), pPst-MP1.8 (*Mlu*I–PstI; nt 22,244–24,116), pPst–HH0.7 (*Hin*dIII–*Hin*dIII; nt 19,054–19,813), pPst-HP4.3 (*Hin*dIII–*Pst*I; nt 19,814–24,116), pPst-NN2.6 (*Nru*I–*Nru*I; nt 20,005–22,652), and pPst-Hc1.7 (*Hin*cII–*Hin*cII; nt 20,129–21,827). A plasmid, phsFlaghisVI$^+$, which contains the FLAG marker peptide sequence and a His_6 tag was constructed in our laboratory using synthetic oligonucleotides. The sequence of the Flag-his portion of this vector starting with the initiation codon and ending in a *Bgl*II site is ATGAGCTCCCGAGACTACAAGGACGACGATGACAAACTCGATCGAGATTCCCGGCATCATCATCATCATCACAGATCT. The plasmid phsFlaghisorf 24VI$^+$ was constructed using the phsFlaghisVI$^+$ plasmid described above and generating the wt *orf24* sequence from Ac*M*NPV by PCR amplification with cloned *Pfu* DNA polymerase (Stratagene, La Jolla, CA) using the primer set: 5′orf24BglII-(GGAAGATCTTTCAACATGTCT TGCATATTAACTGCG), 3′orf24RI-(CCGGAATTCTTATATACTATTTTCTA TTACATATTC). The plasmid phsFlaghisorf 24VI$^+$ was digested with *Bgl*II and *Eco*RI to obtain only the ORF24 coding sequence and cloned under the control of the 6.9K promoter to generate the plasmid, p6.9Korf 24. This plasmid, where ORF24 expression is directed by the 6.9K promoter (Todd *et al.,* 1996), also contains a portion of Ac*M*NPV flanking the *polh* gene and hr5 sequences.

The plasmid ptsB97Pst-I was constructed by cloning the *Pst*I-I fragment (nt 18,880–24,116) from tsB97 viral DNA into the *Pst*I site of pBluescript(KS$^+$) and used to sequence orf24 and flanking regions of the mutant.

Marker rescue experiments and sequencing

All marker rescue experiments were performed using SF-21 cells adapted for growth at 33°C. Cells were plated at a density of 1.8×10^6 cells per 60-mm-diameter tissue culture plate and transfected with tsB97 viral DNA (1 μg) only or tsB97 DNA in combination with a genomic subclone of Ac*M*NPV using 5 μg of Lipofectin reagent (Gibco/BRL, Gaithersburg, MD). Following a 4-h transfection period the cells were overlayed with 0.5% agarose in TC-100 (with serum) and returned to 33°C. Cells were examined after 5 days for the presence of occlusion-positive plaques. The mutation in tsB97 was initally mapped using a library of overlapping genomic clones of Ac*M*NPV described previously (Passarelli and Miller, 1993) followed by the subclones of wt Ac*M*NPV described above which included the *Bam*HI-C, *Pst*I-I, and *Hin*dIII-M fragments.

Sequencing was performed by the Molecular Genetics Instrumentation Facility (University of Georgia) using a Model 373A automated DNA sequencer and the Prism Dye Terminator sequencing kit (Applied Biosystems, Inc., Foster City, CA). A set of primers was designed for sequencing both strands of the pPst-Hc 1.7 clone from wt Ac*M*NPV and the corresponding region from the ts mutant DNA clone, tsB97Pst-I.

Analysis of protein synthesis in infected cells

Metabolic labeling experiments were performed using SF-21 cells adapted for growth at either 23 or 33°C which had been infected at a multiplicity of infection (m.o.i.) of 20 PFU/cell with either wt Ac*M*NPV or tsB97 mutant virus. Cells were plated at a density of 1.0×10^6 cells per 35-mm-diameter tissue culture plate, virus was adsorbed for 1 h at either 23 or 33°C, and at various times postinfection cellular proteins were pulse-labeled using 25 μCi of Tran^{35}S-label (ICN, Irvine, CA; ca. 1000 Ci/mmol) according to the method outlined in O'Reilly *et al.* (1992). Cells were lysed in 1× SDS gel loading buffer (2% SDS, 62.5 mM Tris–HCL, pH 6.7, 15% glycerol, 0.001% bromophenol blue, 0.1 M DTT) supplemented with Protease Inhibitor Cocktail (Pharmingen, San Diego, CA). For analysis of P10 production, cells were lysed in the same buffer and proteins were separated using 15% SDS–PAGE and visualized using Coomassie blue staining. Molecular weight sizes were estimated using the Multimark multicolored protein standard (NOVEX, San Diego, CA).

RNA isolation

Total cellular RNA was isolated from mock-infected, wt Ac*M*NPV-infected, or tsB97-infected SF-21 cells adapted for growth at 33°C. For each time point, two plates of 5×10^6 cells per 100-mm-diameter tissue culture plate were infected at an m.o.i. of 20 PFU/cell and total RNA was obtained according to the method of Chirgwin *et al.* (1979).

Northern blot analysis

Total cellular RNA from mock-infected cells and cells infected with either wt Ac*M*NPV or tsB97 virus at 33°C was denatured by glyoxalation and 25 μg/lane was electrophoresed through 1.2% SeaKem ME agarose (FMC Bioproducts, Rockland, ME) according to the protocol outlined in O'Reilly *et al.* (1992). The marker lane containing the 0.24- to 9.5-kb RNA ladder (Gibco/BRL) was denatured with glyoxal before gel electrophoresis and stained separately with ethidium bromide. The remainder of the gel was transferred onto nylon blotting membrane and hybridized overnight with a specific probe at 42°C in the presence of 50% formamide. All probes were labeled by nick-translation using [$\alpha-^{32}$P]dCTP (3000 Ci/mmol, NEN Research Products, Wilmington, DE). The probe used to detect *polh* transcripts was a gel-purified *Eco*RV–*Kpn*I fragment from the plasmid pRIRV, which is a subclone of right half of *Eco*RI-I from the *Eco*RV site to

*Eco*RI. The probe used to detect *p10* transcripts was generated by labeling plasmid pQP10, which contains all of Ac*M*NPV *Hin*dIII-Q and *Hin*dIII-P fragments. Transcripts of *vp39* were detected using a *Nar*I–*Sca*I fragment of the plasmid pSTSNM, described by Thiem and Miller (1989). A probe for *6.9K* transcripts was made from the plasmid pHD2, containing a *6.9K* cDNA (Wilson *et al.,* 1987). Transcripts specific for the *603 orf* were detected by labeling the plasmid pBSPRI-I, an *Mlu*I–*Eco*RV subclone of Ac*M*NPV *Eco*RI-I fragment.

Production of infectious budded virus from wt- and tsB97-infected cells at 33°C

The rate of release of infectious budded virus was measured by infecting SF-21 cells at an m.o.i of 5 PFU/cell (2×10^6 cells per 60-mm-diameter dish). Virus was initially adsorbed for 1 h at 33°C, after which the virus inoculum was completely removed, the cells were washed three times with 5 ml of media, and a final 5 ml of complete medium was added to the cells. Before returning the cells to the 33°C incubator, 300 μL of medium was removed and stored for subsequent titer determination (this corresponded to the 0-h sample). At 6, 10, 24, 48, and 72 h p.i., 300 μL of medium was removed and reserved at 4°C. Infections with the tsB97 mutant virus were performed at 33°C in duplicate plates and the two samples were titered separately on SF-21 cells adapted for growth at 23°C. All virus samples were titered three times to determine the mean and standard error values. Data were plotted using SIGMA PLOT (Jandel Scientific).

To examine the release of budded virus, cells were infected with either wt or tsB97 virus at 33°C. Some cells were infected in the presence of aphidicolin (Sigma Chemical Co., 5 μg/ml). At 40 h p.i., medium was collected and spun at 1000 rpm for 10 min to remove cells. Medium was collected from mock-infected cells at the same time. Budded virus was collected by spinning through a 25% sucrose solution containing 5 mM NaCl and 10 mM EDTA. Virus samples were resuspended in PBS (pH 6.2) and analyzed by Western analysis using the anti-VP39 polyclonal antibody (Thiem and Miller, 1989).

Electron microscopy of wt- or tsB97-infected SF-21 cells

SF-21 cells (5×10^6 cells per 100-mm dish) were infected at an m.o.i. of 10 PFU/cell and incubated at 33°C. At 16 h p.i., the cells were washed in phosphate-buffered saline (PBS), pH 6.2, and then harvested and pelleted at 1000 rpm for 5 min. The pellets were fixed in 5% glutaraldehyde, 0.1 M tetrabutylammonium fluoride (Aldrich Chemical Co., Milwaukee, WI) in 0.025 M HEPES buffer for 1 h at room temperature. The cells were pelleted and then resuspended in 1% osmium tetroxide in 0.025 M Sorensen's phosphate buffer (pH 7.4) with 0.1 M potassium fluoride (KF). The fixed samples were then dehydrated through a series of increasing concentrations of acetone (50, 70, 80%), each with 0.1 M KF, then a series of acetone (95 and 100%) without KF, followed by 100% propylene oxide. Cell pellets were embedded in Epon 812 and, following sectioning, were stained with 2% uranyl acetate in a solution of sodium citrate and lead citrate. Samples were examined using a JEOL 100CX II transmission electron microscope.

Western blot analyses

SF-21 cells were infected with wt Ac*M*NPV or tsB97 at an m.o.i. of 10 PFU/cell and individual samples were harvested at 6, 12, 24, and 48 h p.i.. For each time point, 1×10^6 cells were lysed in 50 μL of SDS lysis buffer (see above). Protein lysates were resolved using 10% SDS–PAGE and electrophoretically transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA). Blocking solution contained 5% nonfat dried milk, 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20. To analyze the levels of capsid protein, a rabbit polyclonal antibody raised to VP39 (Thiem and Miller, 1989) was used at a dilution of 1:8000 as the primary antibody and a mouse anti-rabbit IgG/HRP conjugate was used for the secondary. For detection of VLF-1 in tsB97-infected cells, the membrane was first incubated with a rabbit polyclonal VLF-1 antibody (S. Yang and L. K. Miller, submitted for publication) at a dilution of 1:2500 and then with a mouse anti-rabbit IgG/HRP conjugate as the secondary antibody (Promega Corp., Madison, WI). For detection of 6.9K, we used an antibody raised to a synthetic peptide (Wilson, 1988) at a dilution of 1:5000 (gift of M.J. Wilson). Proteins were detected using a chemiluminescence detection kit (ECL Kit, Amersham, Life Science, Arlington Heights, IL).

DNA transfections and transient assays

Monolayers of SF-21 cells (2×10^6 cells per 60-mm-diameter dish) were transfected using Lipofectin® Reagent (Gibco/BRL, Gaithersburg, MD) with a combination of 0.5 μg of Ac*M*NPV genomic DNA clones from a library of overlapping clones described previously (Passarelli and Miller, 1993), 2 μg of a CAT reporter plasmid under control of the *polh* promoter (phcwt), and 0.5 μg each of selected DNA clones to express known LEFS. Plasmids used to supply the required LEFs are as follows: pBCNE (*lef-1*), pAcIAP-Ps/NsiI (*lef-6*), pNspAfI (*39K*), pH_3R (*lef-11*), p47 (*p47*) (see Todd *et al.,* 1995). ORF24 was expressed from its native promoter using the plasmid pPstNN 2.6 (designated ORF24) or from the 6.9K promoter using the plasmid p6.9Korf24 (designated 6.9kORF24); both are described above. *vlf-1* was expressed from its native promoter (pXA7), designated VLF-1, or from the 6.9K promoter (pXA76.9), designated 6.9kVLF-1. A plasmid containing a frame shift in *vlf-1* (pXA7ΔBSA), designated VLF-1 fs, served as a negative control. Cells were

harvested 72 h p.i. and CAT assays were performed according to the method of Gorman *et al.* (1982) on 1/2500 of the cleared cell lysate.

Phosphoprotein analysis

A time course was performed to examine protein phosphorylation at 33°C by metabolic labeling of either wt- or tsB97-infected cells. SF-21 cells were infected at an m.o.i. equal to 10 and then at various times p.i. TC-100 was replaced with phosphate-free TC-100 prepared according to the method outlined in O'Reilly *et al.* (1992). Cells were preincubated in phosphate-free medium for 1 h prior to the addition of [^{33}P]orthophosphoric acid (NEX-080, Dupont NEN, Wilmington, DE). Cells were labeled for either 1 h with 20 μCi or 6 h with 10 μCi of $^{33}P_i$. Following the labeling period, cells were washed three times with cold PBS (pH 6.2), pelleted, and lysed in SDS lysis buffer containing 50 mM EDTA. Proteins were resolved by 12% SDS–PAGE and the gels were first stained with Coomassie blue and then subjected to fluorography according to O'Reilly *et al.* (1992).

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